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Photo-Activation of Respiration in Degenerated Sperm of Echiuroid, Oyster and Sea Urchin

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ABSTRACT—In sperm of echiuroid, oyster and sea urchin, which had been incubated in sea water at 20°C for 15 hr, the respiratory rate, which was lower than in fresh sperm, was enhanced by light at 430, 530 and 570 nm more strongly than at the other examined wavelengths. In fresh sperm, respiration was not affected by light. This incubation resulted in marked decrease in the activity of NADH cytochrome ***c*** reductase and the amount of cytochrome ***b*** in these sperm, more evidently than appreciable decrease in the cytochrome ***c*** oxidase activity and the amount of cytochrome ***aa***$_3$. NADH cytochrome ***c*** reductase was activated by light with peaks of photo-activation at 430, 530 and 570 nm in fresh and incubated sperm. A marked decrease in the cytochrome ***b*** amount during incubation probably makes the reactions, such as catalyzed by NADH cytochrome ***c*** reductase, rate-limiting in mitochondrial respiratory chain, to reduce the respiratory rate in incubated sperm. Photo-activation of these enzyme reactions, in which cytochrome ***b*** is involved, seems to enhance the respiratory rate, only when they become rate-limiting. Tunicate sperm, which NADH cytochrome ***c*** reductase was insensitive to light, did not exhibit photo-activation of respiration, even after a long time incubation.

INTRODUCTION

Previously, it has been reported that the respiration, inhibited by CO, is reactivated by light irradiation in sperm of echiuroid, sea urchin and starfish (Fujiwara *et al.*, 1991; Yasumasu *et al.*, 1991) as well as in echiuroid eggs (Tazawa *et al.*, 1991). In the absence of CO, light irradiation does not augment the respiratory rate in these gametes. In sperm of these species and echiuroid eggs, respiration is strongly blocked by antimycin A and CN^- in the presence and absence of CO, either in the dark or under light irradiation. Thus, it seems certain that the respiration in these sperm and eggs, depending on electron transport through mitochondrial respiratory chain, is reactivated by light irradiation, when respiration is strongly inhibited by CO.

In action spectra for photo-reactivation of CO-blocked respiration in these sperm, the peaks for photo-reactivation are found at the same wavelengths to those in absorption spectrum of reduced cytochrome ***b*** but are not found at the wavelengths similar to those in absorption spectrum of CO-bound cytochrome ***aa***$_3$ (Fujiwara *et al.*, 1991; Yasumasu *et al.*, 1991). These suggest that photo-reactivation of respiration in CO-exposed sperm of these species does not always depend on light-induced relief of cytochrome ***c*** oxidase, or cytochrome ***aa***$_3$, from CO-caused inhibition, but results from light-induced augmentation of cytochrome ***b*** reaction (Tazawa *et al.*, 1996a).

It is also found that light irradiation enhances the activities of NADH cytochrome ***c*** reductase and succinate cytochrome ***c*** reductase, in which cytochrome ***b*** is involved, in mitochondria isolated from these sperm (Tazawa *et al.*, 1996b). Photo-activation of these complex enzymes occurs in the absence of CO with peaks of photo-activation at the same wavelengths as in absorption spectrum of cytochrome ***b***. However, photo-activation of respiration in these sperm, with peaks of photo-activation at the wavelengths as above, does not occur in the absence of CO (Fujiwara *et al.*, 1991; Yasumasu *et al.*, 1991). At present, there is no answer for a question of why photo-activation of these complex enzymes, observed in the absence of CO, is not made apparent as an increase in the respiratory rate in these sperm.

It was found in the present study that degeneration of sperm, caused by an incubation for a long time, made their respiration sensitive to light irradiation in echiuroid, oyster and sea urchin.

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MATERIALS AND METHODS

Sperm of echiuroid, oyster, sea urchin and tunicate

Sperm of the echiuroid, *Urechis unicinctus*, the oyster, *Crassostrea gigas*, the sea urchin, *Anthocidaris crassispina*, and the tunicate, *Ciona intestinaris*, were obtained by the procedures reported previously (Yasumasu *et al.*, 1991). Dry sperm of these species were stored in an ice bath until use.

Incubation of sperm

Dry sperm of these species were diluted in ASW (artificial sea water), which had been filtered through Millipore filter, to make the sperm concentration about 10^9 cell/ml and kept at 20°C with occasional agitation in a sterilized glass vessel. An aliquot of sperm suspension, obtained at 5, 10, 15, 20 or 25 hr of incubation, was centrifuged at 1,500g for 10 min. Sperm pellet, washed with filtered ASW, was used to estimate the respiratory rate, the NADH cytochrome ***c*** reductase activity and the cytochrome ***c*** oxidase activity. The numbers of sperm were counted by a light microscope using a hemocytometer.

Estimation of the respiratory rate

Sperm suspension in 2.0 ml, containing about 2×10^9 cells, was placed in a glass vessel and kept at 20°C by circulating temperature-controlled water through water jacket surrounding vessel. This glass vessel was closed with a stopper, which was equipped with an oxygen electrode (Yellow Springs Co., USA). The rate of oxygen decrease in sperm suspension in the closed glass vessel was monitored by an oxygen electrode, being gently stirred by a magnetic stirrer. The respiratory rate, calculated on the basis of oxygen decrease in sperm suspension, is expressed as nmol $O_2/10^9$ cell/min. Stock solutions of NaCN, antimycin A, oligomycin and FCCP (carbonyl-cyanide p-trifluoromethoxyphenylhydrazone), in less than 20 µl were added to 2 ml sperm suspension through a small hole in the stopper. Final concentrations of these compounds are shown in Table 2.

Homogenate and mitochondria fraction

Sperm in 50 ml suspension were harvested by centrifuge at 1,500g for 10 min and washed with ASW, at various times of the incubation at 20°C. Sperm pellet thus obtained was diluted in the homogenizing medium containing 0.5 M sucrose, 10 mM $MgCl_2$ and 10 mM EGTA and was homogenized in a glass homogenizer by motor-driven Teflon pestle in an ice bath. Usually, sperm homogenate was used as the enzyme source for the estimation of cytochrome ***c*** oxidase and NADH cytochrome ***c*** reductase. In some cases, mitochondria fraction was used as the enzyme source. Sperm homogenate was centrifuged at 8,000g for 15 min. The precipitate obtained was suspended in the homogenizing medium and centrifuged at 500g for 10 min. The supernatant was centrifuged again at 8,000g for 15 min. The obtained precipitate was resuspended in the homogenizing medium and used as crude mitochondria fraction. Homogenate and crude mitochondria fraction are adjusted in their concentration to contain 5×10^9 cell eq. (equivalent)/ml. All procedures were performed in the cold.

The cytochrome *c* oxidase activity

The activity was estimated according to the method of Rafael (1983). Reaction mixture was composed of 2 ml phosphate EDTA buffer (50 mM KH_2PO_4, 1 mM EDTA-$Na_2$2H_2O pH 7.2), 40 µl of 50 mg/ml cytochrome ***c***, 10 µl of 10 mM TMPD and 150 µl of 100 mM Na-ascorbate. To 2.2 ml reaction mixture in a closed glass vessel, equipped with an oxygen electrode, sperm homogenate or mitochondria fraction in 100 µl was added through a small hole in the stopper, with which the glass vessel was closed. Oxygen concentration was estimated in the reaction mixture which was gently stirred by a magnetic stirrer. The activity of cytochrome ***c*** oxidase was calculated on the basis of tracing record of the change in oxygen concentration in the reaction mixture containing sperm homogenate or mitochondria fraction. The activity is expressed as nmol O_2 utilized/10^9 cell eq./min.

The activity of NADH cytochrome *c* reductase

The estimation of this enzyme activity was made essentially according to the method of Mahler (1955). The reaction mixture in 2.2 ml was composed of 2 ml 50 mM phosphate EDTA buffer at pH 7.2 containing 10 mM $MgCl_2$, 10 µl of 5 mM NADH, 45 µl of 50 mg/ml cytochrome ***c***, 10 µl of 5 mM KCN and appropriate amount of sperm homogenate or mitochondria. The reaction for 2 min was initiated at 25°C by adding enzyme source to the reaction mixture and was terminated by chilling the mixture in dry ice following cooling in an ice bath for 30 sec. Difference in the absorbance between 550 and 540 nm was estimated by a dual beam two wavelength spectro-photometer (Model 557, Hitachi Co., Tokyo). The amount of reduced cytochrome ***c*** was calculated on the difference in the absorbance and expressed as the amount of O_2 to be utilized for oxidation of reduced cytochrome ***c***. The activity of NADH cytochrome ***c*** reductase is expressed as nmol O_2 eq./10^9 cell eq./min.

Difference spectrum

Difference spectrum between mitochondria suspension reduced by hydrosulfite (adding 10 mg fine powder of hydrosulfite to 2 ml suspension) in the sample side and the suspension oxidized by H_2O_2 (adding 10 µl of 5% H_2O_2 solution) in the reference side was obtained by a dual beam, two wavelengths spectrophotometer (model 557, Hitachi, Co., Tokyo). In this experiment, mitochondria fractions, exhibiting more than 85% of the cytochrome ***c*** oxidase activity in homogenates, were used.

Light irradiation

Light irradiation at various wavelengths between 410 and 600 nm was performed by the Okazaki Large Spectrograph at National Institute for Basic Biology, Okazaki. The light fluence rate, which was altered by neutral density filters, was estimated by a photon density meter HK-1, custom made at the Institute for Physical and Chemical Research, Wako.

Chemicals

NADH, cytochrome ***c***, FCCP, oligomycin and antimycin A were obtained from Sigma Chem. MO., USA. Ascorbate-Na, EDTA (ethylenediamine tetraacetic acid disodium salt), EGTA (O,O'-Bis (2-aminoethyl)ethyleneglycol-N,N,N'-N'-tetraacetic acid), TMPD (tetramethyl para-phenylenediamine), KCN and NaCN were purchased from Kanto Chem., Co., Tokyo. ASW was from Jamarin Lab., Osaka. All other chemicals were of analytical grade.

RESULTS

Fig. 1 shows the change in the respiratory rate in sperm of echiuroid (**A**), sea urchin (**B**), oyster (**C**) and tunicate (**D**) during their incubation at 20°C. The results shown in Fig. 1 are typical among at least 3 experiments made on sperm of these species. During incubation up to 10 hr in sperm of echiuroid and sea urchin and up to 5 hr in sperm of oyster and tunicate, the respiratory rate gradually decreased and was not enhanced by light irradiation at 430 nm. Thereafter, the respiratory rate decreased somewhat steeply in sperm of all examined species and was evidently enhanced by light at 430 nm in sperm of echiuroid (Fig. 1**A**), sea urchin (Fig. 1**B**) and oyster (Fig. 1**C**). Light irradiation was performed on sperm after the rate in the dark had been estimated at the time of incubation shown in Fig. 1. In sperm of these species, incu-

bated for a long time, light irradiation resulted in gradual increase in the respiratory rate for about 1 min and then made the steady rate higher than in the dark. The rates of respiration under light irradiation shown in Fig. 1 were those obtained 2 min after the onset of light irradiation.

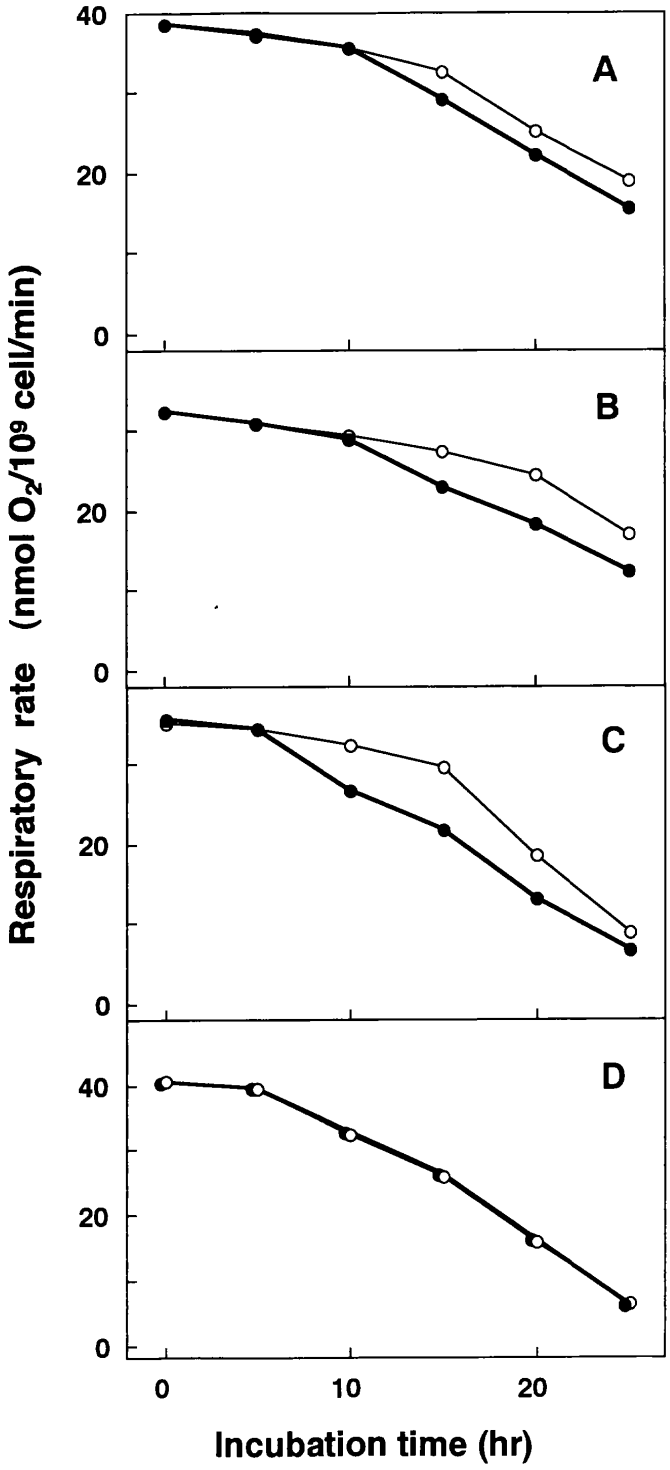

Fig. 1. Change in the respiratory rate in sperm of echiuroid (**A**), sea urchin (**B**), oyster (**C**) and tunicate (**D**) during their incubation at 20°C. Experimental procedures are shown in Materials and Methods. Sperm suspension containing about 10^9 cell/ml was incubated at 20°C for 5, 10, 15, 20 and 25 hr. The respiratory rates in sperm of these species estimated in the dark are shown with solid circles (●) and those under light irradiation at the wavelengths of 430 nm are shown with open circles (○). Light fluence rate was around 100 (98.5–131.6) μ mol/cm^2·s. The values shown are typical among 3–5 experiments.

In several experiments made on other sperm batches, the initiation time of steep decrease in the respiratory rate was not always the same as shown in Fig. 1. For instance, the initiation time of steep decrease was about 5 hr of incubation in 1 among 4 experiments made on echiuroid sperm. In 2 among 5 experiments made on oyster sperm, steep decrease in the respiratory rate was not found up to 10 hr of incubation. Photo-activation of respiration did not occur in these sperm, unless the decrease in the respiratory rate became steep during incubation. In all examined sperm batches of echiuroid, sea urchin and oyster, steep decrease was initiated at a time of incubation between 5 and 10 hr and photo-activation of respiration was evident at 15 hr of incubation. Thus, further studies were made on sperm obtained at 15 hr of incubation, to characterize photo-sensitive respiration in sperm of these species. Hereafter, sperm incubated for 15 hr are referred to, for the convenience, as the incubated sperm. Sperm obtained 15 min after dry sperm dilution, specified as the fresh sperm, were also used to find out difference in the characteristics of respiration between the incubated and fresh sperm. In sperm of tunicate, the respiratory rate decreased during incubation in the same manner as in sperm of other examined species but was not enhanced by light at any time of incubation (Fig 1D). For the comparison, sperm of tunicate incubated for 15 min and 15 hr were also used as the fresh and incubated sperm.

Table 1 shows the respiratory rates in the fresh and incubated sperm. In all examined species, the respiratory rate was lower in the incubated sperm than in the fresh sperm. These values shown in Table 1 were obtained on sperm of other batches than used in the experiments shown in Fig. 1. The values shown in Table 1 are very alike to those shown in Fig. 1. In the fresh sperm of all examined species, the respiratory rate was not altered by light, at all examined wavelengths (Table 1). The incubated sperm of echiuroid, sea urchin and oyster exhibited photo-activation of respiration strongly at 430, 530 and 570 nm and weakly at 400, 490, 550 and 600 nm at around 100 μ mol/cm^2·s. At all examined wavelengths, respiration in the fresh and incubated sperm of tunicate was hardly activated by light irradiation (Table 1).

The percent activation of respiration by light, shown in Table 1, was calculated on the basis of the respiratory rates estimated on sperm in a suspension 2 min before and after the onset of light irradiation at each examined wavelength shown in Table 1. Hence, it took 5 min to estimate the respiratory rate under light irradiation at a certain wavelength, following estimation of the rate in the dark, and about 40 min to obtain the rates before and after the onset of light irradiation at all examined wavelengths. In all experiments, the respiratory rates before and after the onset of light irradiation at 430 nm were also estimated in these sperm at 16 hr of incubation and were confirmed to be practically the same as those obtained at 15 hr of incubation. The respiratory rate in these sperm in the dark did not appreciably decrease in the period of incubation between 15 and 16 hr. Difference in the percent

activation of respiration by light between the wavelengths, shown in Table 1, does not seem to be due to the change in the characteristics of respiratory system in this 1 hr period of incubation.

In the incubated sperm of echiuroid, sea urchin and oyster, percent-activation of respiratory rate by light irradiation at 430 nm was not lower at 50 μmol/cm^2·sec than at about 100 μ mol/cm^2·sec and was evidently low at about 10 μmol/cm^2·sec (data not shown). Activation of respiration by light at 430 nm probably becomes maximum plateau at the light fluence rate higher than about 50 μmol/cm^2·sec. The activation of respiration by light at other examined wavelengths was not so large as at 430 nm even at about 100 μmol/cm^2·sec (Table 1) and was quite low at about 50 μmol/cm^2·sec (data not shown). At the wavelengths other than 430 nm, the higher fluence rate than 100 μmol/cm^2·sec is probably indispensable to make the photo-activation of respiration maximum. Photo-activation occurs with a peak at 430 nm in these incubated sperm. Percent activation of respiration by light at 530 and 570 nm was evidently larger than at 490, 550 and 600 nm (Table 1). Probably, photo-activation of respiration also occurs with peaks at 530 and 570 nm, as well as at 430 nm.

In sperm of these species, either the fresh or incubated ones, the respiration was strongly inhibited by antimycin A and CN^-, under light irradiation or in the dark (Table 2). In sperm of all examined species, respiration certainly results solely from electron transport through mitochondrial respiratory chain. The respiration in sperm of all examined species

Table 1. Photo-activation of respiration in sperm of echiuroid, sea urchin, oyster and tunicate. Respiratory rate was estimated under light irradiation and in the dark in sperm of echiuroid, sea urchin, oyster and tunicate, incubated for 15 min (fresh sperm) and for 15 hr (incubated sperm). Percent activation of respiration by light was calculated in each experiment. The light fluence rate was made around 100 (10 9.6-115.1) μmol/cm^2·s by neutral filters at all examined wavelengthes. Each value is the mean±SEM for 3 experiments.

	Respiratory rate (nmol O_2/10^9 cell/min) Dark	Percent activation of respiration by light (%) Wavelength (nm)						
		400	430	480	530	550	570	600
Echiuroid								
freshsperm	37.4 ± 1.75	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
Incubated sperm	28.0 ± 1.47	2.36 ± 1.43	20.2 ± 3.09	0.8 ± 0.7	6.86 ± 0.25	2.83 ± 1.44	12.9 ± 3.06	3.20 ± 0.51
Sea urchin								
fresh sperm	31.4 ± 1.03	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
Incubated sperm	23.4 ± 2.22	5.87 ± 3.84	22.9 ± 4.60	2.67 ± 2.02	15.6 ± 4.50	2.47 ± 1.16	18.5 ± 3.96	2.87 ± 0.56
Oyster								
fresh sperm	35.6 ± 1.69	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
Incubated sperm	21.8 ± 1.25	7.70 ± 0.8	33.6 ± 8.68	4.30 ± 3.0	19.4 ± 3.54	6.77 ± 2.29	21.7 ± 2.04	7.20 ± 1.93
Tunicate								
fresh sperm	40.2 ± 2.06	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
Incubated sperm	26.6 ± 1.80	< 0.1	< 0.2	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1

Table 2. Effects of AMA. CN^-, FCCP and oligomycin on the respiration in sperm incubated for 15 min and 15 hr at 20°C. Sperm incubated for 15 min at 20°C were shown as fresh sperm. Light irradiation at 430 nm was made at the light fluence rate around 150 (147.7–169.6) μmol/cm^2·s. Values shown are typical among 2–4 experiments.

Sperm			Respiratory rate (nmol/cm^2·s) None	AMA (20 μg/ml)	CN^- (0.1 mM)	FCCP (5 μM)	Oligomycin (10 μM)	Oligomycin +FCCP
Echiuroid	fresh	dark	38.5	13.7	< 0.2	38.9	2.9	39.2
		light	38.9	12.8	< 0.2	38.7	1.4	39.1
	Incubated	dark	29.2	7.0	< 0.2	29.3	2.7	29.9
		light	35.1	6.5	< 0.2	35.8	4.1	35.1
Sea urchin	fresh	dark	32.4	7.7	< 0.2	33.8	5.4	33.7
		light	32.6	7.0	< 0.2	33.7	3.1	34.2
	Incubated	dark	23.0	3.8	< 0.2	24.1	3.4	25.2
		light	27.4	1.6	< 0.2	28.3	3.4	28.1
Oyster	fresh	dark	35.5	9.7	< 0.2	35.1	3.6	36.2
		light	35.1	9.0	< 0.2	35.3	3.1	35.6
	Incubated	dark	21.8	5.8	< 0.2	22.1	4.7	22.0
		light	30.6	6.5	< 0.2	31.0	4.1	30.6
Tunicate	fresh	dark	40.3	2.7	< 0.2	40.5	3.1	39.4
		light	40.7	3.2	< 0.2	40.1	3.8	40.3
	Incubated	dark	26.1	2.7	< 0.2	26.8	3.2	27.0
		light	25.7	4.7	< 0.2	25.6	1.8	25.7

was not affected by FCCP but was strongly blocked by oligomycin, under light irradiation or in the dark (Table 2). FCCP released oligomycin-caused inhibition of respiration. Respiration in these sperm seems to be coupled to oxidative phosphorylation but is not under acceptor-control, probably because of high concentration of available ADP. Light-induced activation of respiration in the incubated sperm of echiuroid, oyster and sea urchin does not seem to be due to change in accep-

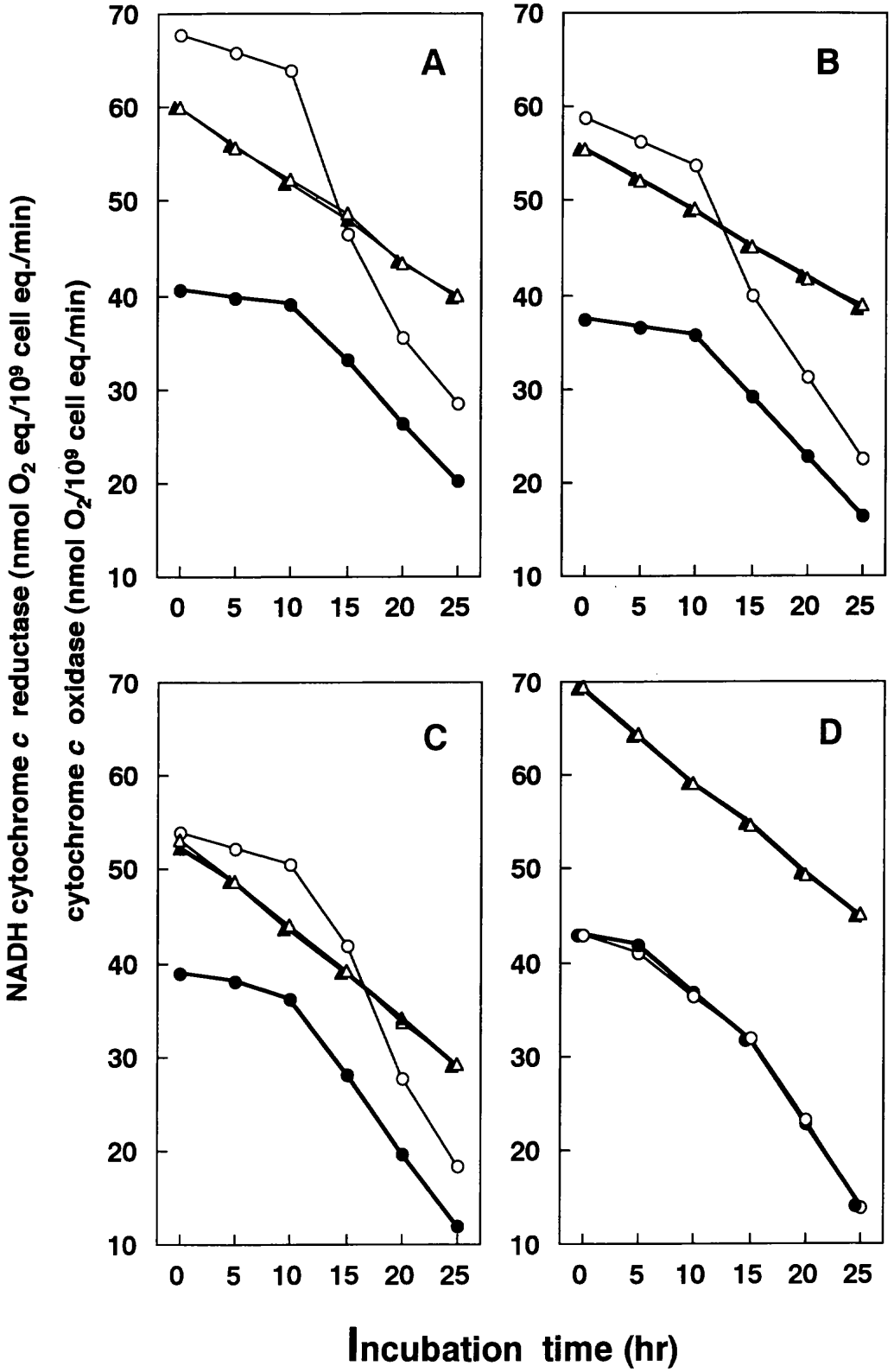

Fig. 2. Change in the activities of NADH cytochrome *c* reductase and cytochrome *c* oxidase in sperm of echiuroid (**A**), sea urchin (**B**) oyster (**C**) and tunicate (**D**) during incubation at 20°C. Experimental procedures are shown in Materials and Methods. Sperm suspensions used were those in experiments shown in Fig. 1. The activities of NADH cytochrome *c* reductase (●, ○) and cytochrome *c* oxidase (▲, △) in sperm homogenate were estimated in the dark (●, ▲) and under light irradiation (○, △) at 430 nm at the light fluence rate of around 100 (110.7–124.6) μ mol/cm^2·sec.

tor-control of respiration.

Fig. 2 shows the activities of cytochrome ***c*** oxidase and NADH cytochrome ***c*** reductase in sperm homogenate obtained at the indicated times of incubation. The activity of cytochrome ***c*** oxidase in sperm of echiuroid (**A**), sea urchin (**B**), oyster (**C**) and tunicate (**D**), constantly decreased during whole span of incubation. The NADH cytochrome ***c*** reductase activity became slightly low at 10 hr of incubation in sperm of echiuroid and sea urchin and at 5 hr in oyster and tunicate sperm. Thereafter, the activity of this complex enzyme decreased somewhat steeply in sperm of all examined species (Fig. 2). The experiments shown in Fig. 2 were made on sperm batches, on which the results shown in Fig. 1 were obtained. During incubation of these sperm, steep decrease in the respiratory rate (Fig. 1) occurs simultaneously with strong decrease in the activity of NADH cytochrome ***c*** reductase (Fig. 2). In some experiments made on these sperm of other batches than shown in Figs. 1 and 2, the time of incubation for initiation of steep decrease in the respiratory rate was not the same as the time shown in figures, but steep decrease in the NADH cytochrome ***c*** reductase was always accompanied by a marked decrease in the respiratory rate. The same was the case in the experiments in which the activity of succinate cytochrome ***c*** reductase was estimated in place of the NADH cytochrome ***c*** reductase activity (data not shown).

In sperm of echiuroid (Fig. 2**A**), sea urchin (Fig. 2**B**) and oyster (Fig. 2**C**), light irradiation at 430 nm at about 100 μ mol/cm^2·sec enhanced the activity of NADH cytochrome ***c*** reductase at all examined times of incubation. In sperm of tunicate, the activity of NADH cytochrome ***c*** reductase was hardly enhanced by light during whole span of incubation. Light irradiation did not alter the activity of cytochrome ***c*** oxidase in sperm of all examined species. Table 3 shows the means of these enzyme activities in the dark and under light irradiation at 430 nm at around 100 μ mol/cm^2·sec in mitochondria isolated from the fresh and incubated sperm of different 3 batches. The results obtained on mitochondria (Table 3) are in well agreement with those obtained on sperm homogenate (Fig. 2).

Fig. 3 shows action spectra for photo-activation of NADH cytochrome ***c*** reductase in mitochondria isolated from sperm of echiuroid (**A**), sea urchin (**B**), oyster (**C**) and tunicate (**D**). The activity of NADH cytochrome ***c*** reductase under light irradiation at 50 μ mol/cm^2·sec, shown in Fig. 3, was calculated on the basis of the relationships between the light fluence rate and the activity of this complex enzyme (data not shown). At the fluence rate slightly higher that 50 μ mol/cm^2·sec, light irradiation at 430 nm induced maximum photo-activation of this complex enzyme, as reported previously (Tazawa *et al.*, 1996b). The sensitivity of this complex enzyme to light seems to be alike to that of respiration. In all examined species, this complex enzyme activity in mitochondria isolated from the fresh sperm was higher than in those isolated from the incubated sperm. Action spectra obtained on mitochondria fractions isolated from the incubated sperm of echiuroid, sea urchin and oyster showed peaks of photo-activation of NADH cytochrome ***c*** reductase at 430, 530 and 570 nm in the same manner as in those isolated from the fresh sperm (Fig. 3). In mitochondria fraction obtained from tunicate sperm (Fig. 3**D**), light irradiation hardly exerted any effect on the activity of NADH cytochrome ***c*** reductase. As also shown in Fig. 3, the activity of cytochrome ***c*** oxidase in mitochondria isolated from the fresh and incubated sperm of echiuroid (**A**), sea urchin (**B**), oyster (**C**) and tunicate (**D**) was not affected by light at the wavelength between 410 and 600 nm at around 100–150 μ mol/cm^2·sec. These action spectra obtained on mitochondria

Table 3. Effect of light on NADH cytochrome *c* reductase and cytochrome *c* oxidase in mitochondria isolated from sperm of echiuroid, sea urchin, oyster and tunicate, incubated for 15 min (fresh sperm) and 15 hr (incubated sperm). Table shows the enzyme activities in the dark and under light irradiation at 430 nm at around 100 (99.2–108.6) μmol/cm^2·s. Percent activation of enzyme by light irradiation, calculated in each experiment, is shown in parenthesis. Recovery of cytochrome c oxidase in mitochondria used in these experiments was more than 86.5%. Values shown are means ± SEM.

	NADH cytochrome ***c*** reductase (nmol O_2 /10^9 eq./min)			
Fresh sperm	Echiuroid	Sea urchin	Oyster	Tunicate
Dark	39.5 ± 0.70	36.0 ± 1.90	35.9 ± 3.05	44.8 ± 4.69
Light	57.8 ± 6.19	51.7 ± 3.51	51.9 ± 3.66	44.7 ± 4.88
Percent activation by light	(46.2 ±13.7)	(43.5 ± 7.31)	(42.6 ± 7.23)	(–0.26 ± 0.66)
Incubated sperm				
Dark	30.3 ± 1.80	27.5 ± 1.23	25.4 ± 0.96	32.0 ± 1.42
Light	40.8 ± 3.89	38.5 ± 2.93	36.9 ± 4.09	32.0 ± 1.42
Percent activation by light	(34.5 ± 9.92)	(40.5 ±12.4)	(45.4 ± 2.83)	(0.14 ± 1.03)
	Cytochrome ***c*** oxidase (nmol O_2/10^9 cel eq./min)			
Fresh sperm	Echiuroid	Sea urchin	Oyster	Tunicate
Dark	50.5 ± 3.07	48.9 ± 1.24	50.8 ± 1.36	58.6 ± 2.38
Light	49.9 ± 3.16	49.3 ± 1.27	50.3 ± 2.47	58.4 ± 2.10
Percent activation by lgith	(–0.14 ± 0.34)	(0.70 ± 1.63)	(–0.97 ± 2.74)	(–0.33 ± 0.83)
Incubated sperm				
Dark	42.6 ± 6.15	36.9 ± 4.48	34.6 ± 3.51	50.5 ± 1.98
Light	42.7 ± 6.22	36.9 ± 4.60	34.7 ± 3.45	50.6 ± 2.21
Percent activation by light	(0.39 ± 0.54)	(1.08 ± 1.59)	(0.12 ± 0.68)	(0.25 ± 0.83)

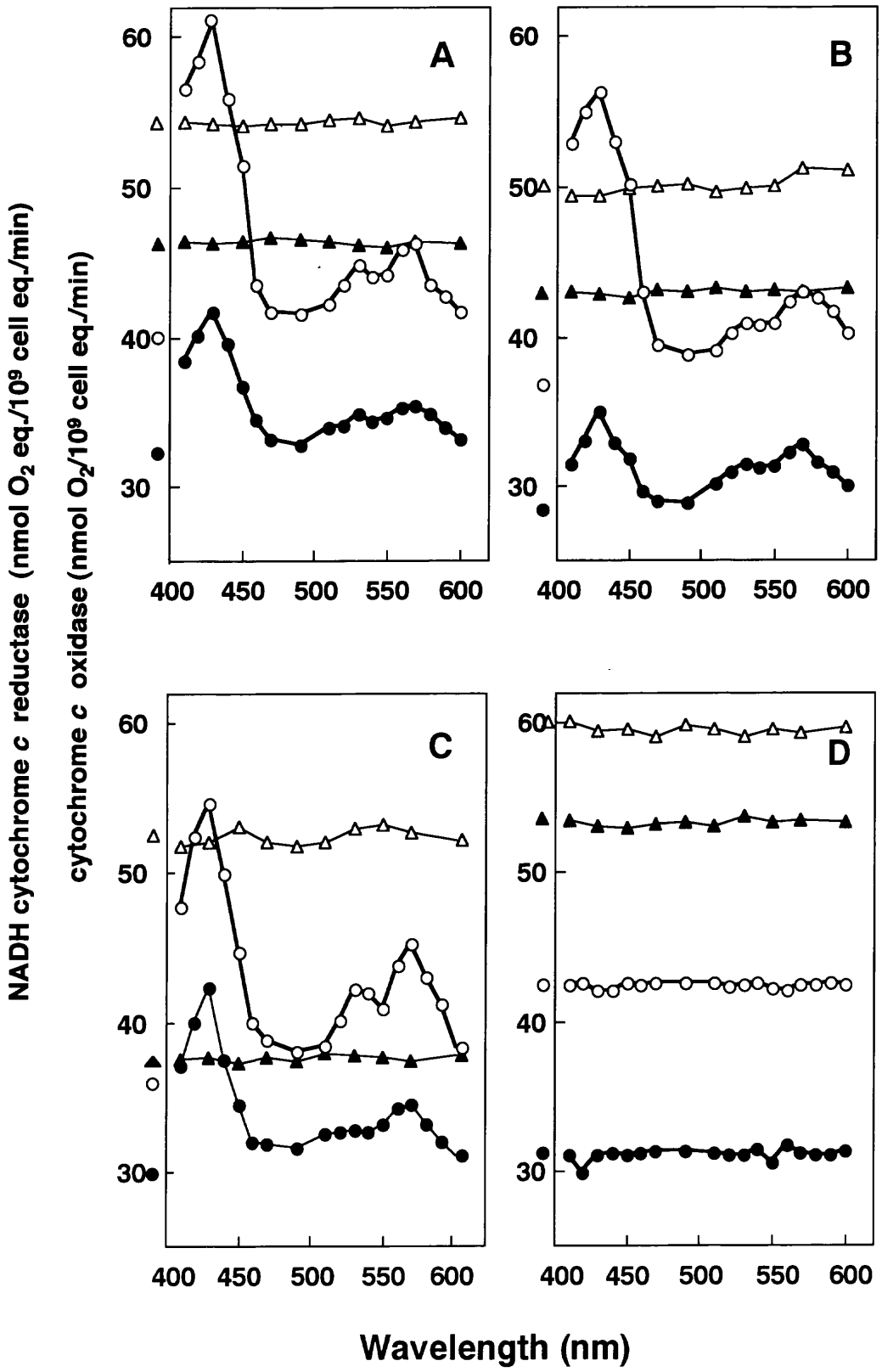

Fig. 3. Action spectra for photo-activation of NADH cytochrome ***c*** reductase in mitochondria isolated from sperm of echiuroid (**A**), sea urchin (**B**), oyster (**C**) and tunicate (**D**). Activity of NADH cytochrome ***c*** reductase under light irradiation at the wavelengths between 420 and 600 nm at the light fluence rate of 50 μ mol/cm^2·sec was calculated on the basis of relationship between the light fluence rate and NADH cytochrome ***c*** reductase at each wavelength (data not shown). NADH cytochrome ***c*** reductase activities under light irradiation are shown with open circles (○) for mitochondria isolated from fresh sperm and with solid circles (●) for those isolated from sperm incubated for 15 hr at 20°C. Cytochrome ***c*** oxidase activities in mitochondria estimated under light irradiation are shown with open triangles for fresh sperm (△) and solid ones for those incubated for 15 hr at 20°C (▲). Activities of these enzyme in the dark are shown in ordinate with the same symbols as those for the activities under light irradiation. These are typical among 3 experiments. Action spectra shown were obtained on mitochondria fraction which showed more than 80% of cytochrome ***c*** oxidase activity in sperm homogenate.

isolated from these sperm are essentially the same to those obtained on sperm homogenates (data not shown).

Difference spectra between hydrosulfite-reduced and H_2O_2-oxidized suspensions of mitochondria, isolated from the fresh sperm of echiuroid (Fig. 4**A**), sea urchin (Fig. 4**B**), oyster (Fig. 4**C**) and tunicate (Fig. 4**D**), showed the peaks of cytochrome ***aa***$_3$, cytochrome ***b*** and cytochrome ***c***. In the incubated sperm of these species, the peaks of cytochrome ***b*** were not so evident as in the fresh sperm (Fig. 4**E–H**). Peaks of cytochrome ***aa***$_3$ and cytochrome ***c*** in mitochondria isolated from the incubated sperm were somewhat poor (Fig. 4**E–H**) but were also as evident as in the fresh sperm of these species (Fig. 4**A–D**). The values of the absorbance in α peaks of cytochrome ***aa***$_3$, cytochrome ***b*** and cytochrome ***c*** in mitochondria isolated from sperm were decreased at 15 hr of incubation by 6.89 ± 0.41, 18.9 ± 3.10 and 2.9 ± 0.27% for echiuroid sperm, 9.23 ± 0.22, 21.6 ± 2.6 and 1.62 ± 0.33% for sea urchin sperm, 4.32 ± 1.64, 34.6 ± 3.2 and 1.95 ± 0.49% for oyster sperm and 2.94 ± 1.04, 15.9 ± 4.7 and 2.16 ± 1.10% for tunicate sperm, (the means ± SEM for 3 experiments), respectively. The absorbance obtained prior to adding hydrosulfite and H_2O_2 to mitochondria suspensions in the sample and reference side was regarded as zero in the absorbance. The absorbance of cytochrome ***b*** decreased more largely than the absorbance of other cytochromes.

In preliminary experiments, effect of light irradiation on the motility of sperm was examined after 15 hr incubation of sperm in the dark. Sperm motility was examined by light microscopy under dark field illumination by light at the wavelengths larger than 700 nm, obtained by a filter. Sperm movement was analyzed on video-records. In the fresh sperm of echiuroid, sea urchin and oyster, the numbers of immotile sperm were quite small in the dark and did not seem to be reduced by light irradiation at 430 nm at the fluence rate of about 100 μmol/cm^2·sec. At 15 hr of incubation in the dark, the numbers of immotile sperm were large, though appreciable numbers of sperm were still motile in all examined species, as reported in sea urchin sperm (Ohtake *et al.*, 1996). Within 3 min after the onset of light irradiation at 430 nm at the fluence rate of about 100 μmol/cm^2·s , the numbers of immotile sperm were reduced by 38.4 ± 9.8% for echiuroid, 41.0 ± 12.6% for sea urchin and 56.6 ± 20.3% for oyster (the mean ± SEM for 3 experiments). The numbers of immotile sperm did not change in the dark at least for 30 min. This indicates that photo-reactivation of sperm motility occurs in sperm of these species. At other wavelengths, photo-reactivation of sperm movement was not so evident as at 430 nm. Relationships between the sperm movement and the light fluence rate at various wavelengths will be published elsewhere.

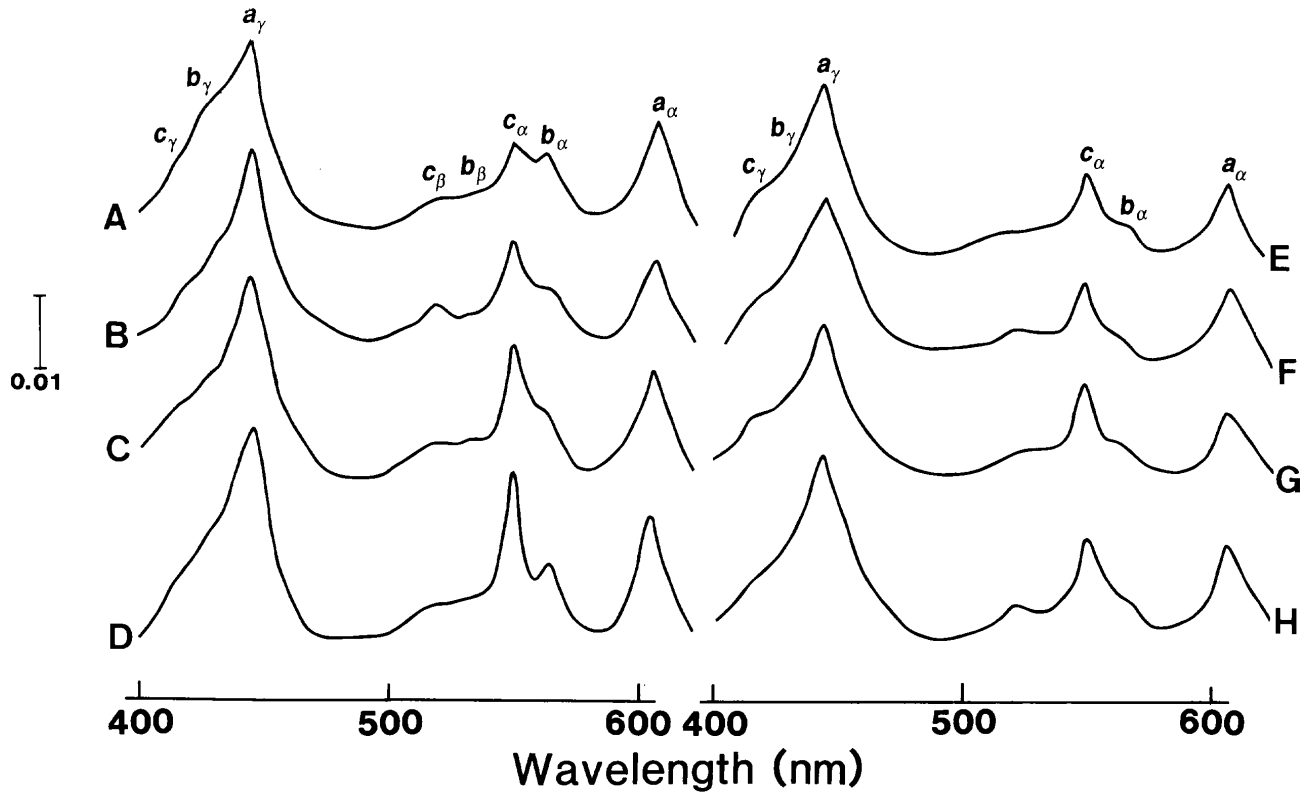

Fig. 4. Difference spectra between hydrosulfite-reduced and H_2O_2-oxidized mitochondrial suspensions isolated from sperm of echiuroid, sea urchin, oyster and tunicate. Difference spectra were obtained on suspensions containing 10^{10} cell equivalent/ml of mitochondria, isolated from sperm of echiuroid (**A**, **E**), sea urchin (**B**, **F**), oyster (**C**, **G**) and tunicate (**D**, **H**). Mitochondria were isolated from fresh sperm (**A–D**) and sperm incubated for 15 hr at 20°C (**E–H**). Recovery of mitochondria, judged on the basis of cytochrome ***c*** oxidase, was at least 86.6%. Peaks of cytochromes are specified with $\boldsymbol{a}_\alpha$, $\boldsymbol{a}_\gamma$ for cytochrome ***aa***$_3$, $\boldsymbol{b}_\alpha$, $\boldsymbol{b}_\beta$, $\boldsymbol{b}_\gamma$ for cytochrome ***b*** and $\boldsymbol{c}_\alpha$, $\boldsymbol{c}_\beta$, $\boldsymbol{c}_\gamma$ for cytochrome ***c***. Vertical bar shows 0.01 in absorbance.

DISCUSSION

Recently, we reported that the activities of NADH cytochrome ***c*** reductase and succinate cytochrome ***c*** reductase are evidently enhanced by light irradiation in mitochondria isolated from gametes and viscera of abalone, echiuroid, oyster and sea urchin but are insensitive to light in mitochondria isolated from fish sperm, (Tazawa *et al.*, 1996b). In the present study, light irradiation was found to exert no effect on NADH cytochrome ***c*** reductase in mitochondria isolated from tunicate sperm as those from fish sperm. It is expected that light-induced activation of these complex enzymes in sperm of abalone, echiuroid, oyster and sea urchin accelerates cytochrome ***c*** reduction to enhance the respiratory rate. However, the respiratory rate in sperm of echiuroid, oyster and sea urchin is not enhanced by light irradiation in the same manner as in tunicate sperm (Fujiwara *et al.*, 1991; Yasumasu *et al.*, 1991).

In sperm of echiuroid, oyster and sea urchin, which had been incubated for a long time (more than 15 hr), light irradiation induced appreciable augmentation of respiration at the wavelengths of 430, 530 and 570 nm more evidently than at other examined wavelengths. NADH cytochrome ***c*** reductase in the fresh and incubated sperm of these species was also activated by light with peaks of photo-activation at 430, 530 and 570 nm. The light fluence rate enough to augment respiration in the incubated sperm was similar to the fluence rate for photo-activation of this complex enzyme. Light irradiation did not enhance the respiratory rate in the incubated sperm of tunicate, which NADH cytochrome ***c*** reductase was not activated by light. Thus, photo-activation of NADH cytochrome ***c*** reductase, seems to become apparent as an increase in the respiratory rate in sperm of these species, when they were incubated for a long time.

In sperm of these species, the decrease in the NADH cytochrome ***c*** reductase activity during incubation was made steep at the time of incubation between about 5 and 10 hr, whereas the decrease in cytochrome ***c*** oxidase was not large even after this time of incubation. Though the initiation time of steep decrease in the NADH cytochrome ***c*** reductase activity during incubation differs among sperm batches, steep decrease in this complex enzyme activity occurred simultaneously with a marked decrease in the respiratory rate. The reaction catalyzed by NADH cytochrome ***c*** reductase seems to become rate-limiting in electron transport through whole span of mitochondrial respiratory chain to reduce the respiratory rate, when this complex enzyme activity becomes quite lower than the activity of cytochrome ***c*** oxidase in sperm incubated for more than 15 hr. Photo-activation of NADH cytochrome ***c*** reductase in sperm of echiuroid, sea urchin and oyster probably becomes apparent as an increase in the respiratory rate, when the reaction catalyzed by this enzyme is made rate-limiting.

During incubation of these sperm up to the initiation time of steep decrease in the NADH cytochrome ***c*** reductase activity, the reaction catalyzed by NADH cytochrome ***c*** reductase is regarded to be not rate-limiting and hence, cytochrome ***c*** reduction in the reaction catalyzed by NADH cytochrome ***c*** reductase is to be enough to make cytochrome ***c*** oxidase reaction close to its V_{max}. In initial period of incubation, the reaction catalyzed by cytochrome ***c*** oxidase in sperm is probably rate-limiting in mitochondrial respiratory chain. Cytochrome ***c*** oxidase was not activated by light in the fresh and incubated sperm of all examined species. The reaction catalyzed by cytochrome ***c*** oxidase does not seem to be responsible for photo-activation of respiration in the incubated sperm of echiuroid, sea urchin and oyster, even when this reaction is rate-limiting in sperm of these species.

In mitochondria, cytochrome ***c*** is not only reduced at the expense of electron equivalent in NADH but also in other electron donors, such as succinate, acyl CoA and glycerol phosphate. Electron equivalent in these electron donors is known to be transported to flavoproteins, then cytochrome ***b*** and cytochrome ***c*** in mitochondrial respiratory chain. The capacities of electron transport from NADH and succinate, are qualitatively estimated as the activities of NADH cytochrome ***c*** reductase and succinate cytochrome ***c*** reductase in mitochondria. These enzymes are augmented by light with peaks of their photo-activation at 430, 530 and 570 nm in sperm of echiuroid, sea urchin and oyster, (Tazawa *et al.*, 1996b). The peaks in action spectra for photo-activation of these enzymes are the same as found in absorption spectrum of reduced cytochrome ***b***. Probably, absorption of photon energy by cytochrome ***b*** augments redox reaction in this cytochrome. Photo-activation of respiration in the incubated sperm of these species occurred with the peaks at the wavelengths as above. Photo-activation of cytochrome ***b*** reaction probably augments the respiratory rate in these sperm when cytochrome ***b*** reaction is made rate-limiting in mitochondrial respiratory chain.

Difference spectra indicated that the amount of cytochrome ***b*** decreased more largely than the decrease in the amount of cytochrome ***aa***$_3$, cytochrome ***c*** oxidase, during 15 hr of incubation in sperm of all examined species. Decrease in the amount of cytochrome ***b*** during incubation, probably due to its degeneration, certainly reduces the activities of enzymes, such as NADH cytochrome ***c*** reductase, and succinate cytochrome ***c*** reductase, in which cytochrome ***b*** is involved. Thus, it is certain that the redox reaction in cytochrome ***b*** becomes lower in its rate than the reaction catalyzed by cytochrome ***c*** oxidase and is made rate-limiting in mitochondrial electron transport in degenerated sperm of all examined species. Sperm, having photo-sensitive cytochrome ***b***, probably exhibit photo-activation of respiration, when the redox reaction in cytochrome ***b*** is made rate-limiting in mitochondrial respiratory chain by degeneration of cytochrome ***b***.

In a previous paper (Tazawa *et al.*, 1996a), it is postulated that probable inhibition of complex enzymes containing cytochrome ***b*** by CO results in photo-reactivation of CO-inhibited respiration in sperm, in which the activities of these complex enzymes are enhanced by light. Inhibition of these enzymes by CO causes decrease in the rate of reaction catalyzed by these complex enzymes to make the reduction of

cytochrome ***c*** rate-limiting in mitochondrial respiratory chain. In CO-exposed sperm, photo-activation of cytochrome ***b*** reaction probably becomes apparent as an increase in the respiratory rate, if CO-caused inhibition is not so strong as causing complete inhibition of cytochrome ***b*** reaction.

It is expected that photo-reactivation of respiration activates sperm movement in the incubated sperm of these species. As expected, sperm movement was found, in preliminary experiments, to become quite weak up to 15 hr of incubation in the dark and was reactivated by light at 430 nm. In strict meaning, this observation is not always the evidence indicating the relationship between photo-reactivation of respiration and sperm movement in the incubated sperm, unless action spectrum for photo-reactivation of sperm movement is confirmed at least to be identical to those for photo-reactivation of respiration. Photo-reactivation of sperm movement is now under investigation.

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